Root hairs shape microbime structure and network interactions upon drought stress

Zhenghong Wang[1#], Zewen Li[1#], Jingxuan Zhai, Xianli Tang[1], Kaixiang Guan, and Yi Song[1*]

Authors:

[1]Institute of Plant and Food Science, Department of Biology, Southern University of Science and Technology (SUSTech), Shenzhen, Guangdong 518055, China

*Correspondence: Yi Song (songy3@sustech.edu.cn).

[#]Contributed equally.

Abstract

Drought is one of the most serious abiotic stresses which also shifts the composition of root associated microbiomes. However, there is a lack of genetic evidence regarding whether and how plant genetic effects positively reshape drought induced microbiome changes. Root hairs play essential roles in water uptaking, but whether root hairs also orchestrate microbiome re-shaping process during drought stress is unknown. By utilizing genetic mutants with enhanced or decreased root hair densities, we detected a significant effect of plant genetic effect on drought induced microbiome changes. In addition, the hairy mutant (*gl2*) triggers a deterministic dominant process during drought induced microbiome re-assembly, which further confirms the involvement of host effects in re-shaping drought induced microbiome changes. *Rhizobiaceae* strains were detected as key biomarker species positively correlated with root hair densities. Moreover, the *gl2* mutant also shapes more complex microbiome co-occurrence networks, with more *Rhizobiaceae* hubs. Our findings unveil the novel roles of root hairs in shaping microbiome structure and network interactions upon drought stress, particularly through regulating the abundance and network centrality of *Rhizobiaceae* strains. Root hair related mutants also broadly affect root metabolome upon drought stress. Understanding the physiological and microbial ecological basis of host mediated microbiome re-shaping under drought helps develop microbiome engineering approaches to combat climate changes.

Introduction

Plant roots are surrounded by highly diverse soil microbial communities, and their co-evolution is crucial for the function of rhizophore ecosystem and plant fitness[1-4]. Different plant ecotypes or cultivars share common and sometimes heritable, microbiome features[5-7], indicating that hosts can selectively shape a "core microbiome". Additionally, plants can even actively reshape the microbiome in response to various stresses to enhance fitness[8]. However, our understanding about the regulations between genes and microbiome ecosystems, and their consequences for plant fitness, are still in its infancy. Drought stress causes significant threat to global agricultural production, with estimates indicating that over 50% of arable land will be affected by drought by 2050[9]. Drought also profoundly disrupts soil microbiomes and plant-associated microbiomes across various plant

species and ecosystems[10,11], and prolonged drought stress even dampens rhizosphere ecosystem resilience after drought recovery[12]. Currently, it remains largely elusive how host reshapes root associated microbiomes under drought stress.

Numerous studies indicate host effects are positively involved in reshaping drought induced root microbiome changes, although we still lack solid genetic evidence to support this. Drought stress exerts a much stronger influence on the composition of root-associated microbiomes than that on bulk soil microbiomes[10]. Furthermore, the effect of drought on root-associated microbiomes varies in different plant development stages (flowering or not)[13], indicating the impact of plant developmental stages on drought-induced microbiome reshaping. Integrated multi-omics approaches have suggested that glycerol 3-phosphate (G3P) and iron are potential metabolic cues affecting drought induced microbiomes shifts[13,14]. Studies in different wild species and crops have demonstrated that drought stress can enrich certain microbes, such as actinobacteria (especially *Streptomyces*), which may enhance host drought tolerance[12,15,16]. Recent research has also shown that tree seedlings infected with different microbiomes from dry or warmer environments exhibit enhanced fitness over multiple years[4]. These studies suggest that microbiome engineering or "microbiome breeding" approaches hold promise as environmentally friendly way to help plants adapt to climate changes[17]. Further genetic studies could provide deeper mechanistic understanding of how host positively regulates drought-triggered microbiome changes, which is crucial for harnessing microbiome to combat drought stress[18].

Plant genetic studies have provided valuable insights into how plants regulate microbiome composition. For example, our previous genetic screening identified a receptor-like kinase, FERONIA, which can regulate the colonization of beneficial Pseudomonads[19], which might be related to pathogen triggered recruitment of beneficial Pseudomonads. A pioneering genetic study demonstrated that *Arabidopsis* mutants disrupting several hormone signaling pathways could alter microbiome structure[20]. This provided the first solid genetic evidence that plants can shape root-associated microbiome. Moreover, by using quadruple mutant to dampens multiple plant immune pathways, a previous study revealed the critical roles of innate immunity in maintaining microbiome homeostasis and plant health[21,22]. Leveraging the power of genetic manipulations in *Arabidopsis*, researchers have further revealed that various plant signaling pathways influence microbiome structure, including epigenetic modifications[23,24], small RNA generating[25] and diverse metabolic pathways[26,27]. Although plant genetic tools have been extensively employed to dissect the regulation mechanisms of microbiome composition and changes, genetic studies regarding how plants positively reshape microbiomes under drought stress are still lacking.

Root hairs are essential for water and nutrient uptake, and also serve as the frontline cells of host-microbiome interactions[28,29]. Additionally, plants secrete approximately 20-30% of their

photosynthetic carbon source as root exudates into the rhizosphere, with root hairs playing a vital role in this process[30]. We thus hypothesized that root hairs might orchestrate microbiome sculpting upon drought stress during long-term evolution. The well characterized genetic regulation pathways of root hair development enable us to obtain mutants with altered root hair densities to study their effects on drought induced microbiome changes. The master transcription factor GLABRA 2 maintains a non-hair cell fate and negatively regulates root hair initiation and development[31]. In contrast, a group of basic helix-loop-helix transcription factors, *ROOT HAIR DEFECTIVE 6* (*RHD6*) and its homolog gene *RHD6-LIKE 1* (*RSL1*), positively regulate the expression of *RSL2-5* genes to promote root hair development[32]. We thus utilized genetic mutants with different root hair densities to dissect the role of root hairs in regulating drought induced microbiome changes. The objectives of this study are: 1) to decipher the effect of genetic mutations related to root hairs on microbiome composition under drought stress; 2) to identify keystone taxa or microbes influenced by root hairs under drought stress; 3) to profile potential metabolic cues related to root hair-mediated community assembly; 4) to validate the effects of root hair-regulated microbes on plant growth and drought tolerance. This study will enhance our understanding of the genetic and physiological mechanisms involved in plant mediated reshaping of microbiome under drought.

Results

Mutants with different root hair densities shift microbiome composition under drought stress

To provide genetic evidence about the role of root hairs in shaping drought induced microbiome changes, we employed the *rsl2 rsl4*[33] double mutant (complete loss of root hairs), the *gl2*[34] mutant (significantly increased root hair density), and wild type *Arabidopsis* for our microbiome profiling analysis (Fig. 1a). In order to mimic a highly diverse natural soil microbiome and enrich drought adapted soil microbes, we mixed natural soils from a tropical rainforest soil (expected to have high microbial diversity) and a dry hot valley in southwest China (expected to have drought-adapted microbes) as our experimental natural soil. To more thoroughly reflect microbiomes changes in different compartments, we sampled both root (thoroughly washed roots) and rhizosphere (closely attached soil on root surface) microbiome samples for each group in our study.

A total of 15,332,970 reads were obtained from 70 samples, including rhizosphere, root, and bulk soil (soil under same treatment procedure without plants) samples (Supplementary Table 1). After filtering, denoising, chimaera removal, and taxonomic annotation (based on the SILVA database using a pre-trained naive Bayes classifier) using DADA2[35], we obtained 5174 amplicon sequence variants (ASVs) from all samples (Supplementary Table 2). We observed significant differences in microbiome composition between root-associated microbiomes and bulk soil (BS) samples at both phylum and family levels, indicating a clear rhizosphere effect on microbiome composition (Supplementary Fig. 1a, b). We found that *gl2* show enhanced alpha diversity of root microbiomes

of samples from the control (Supplementary Fig. 1c), suggesting a positive role of root hairs in maintaining microbial diversity.

Principal coordinated analysis (PCoA) based on Bray–Curtis dissimilarities were performed for all samples. Our results showed that samples belonging to different compartments (like bulk soil, root, and rhizosphere) clustered in distinct groups (Fig. 1b; R^2=0.4757, P <0.001), as well as a significant separation among treatments (Fig. 1b; R^2 = 0.039*, P <0.05). Both drought stress and root compartments (root and rhizosphere) had substantial effects on microbiome composition, consistent with previous reports[36,37]. Interestingly, we found that plant genotypes also exert significant influence on microbiome compositions (Fig. 1b; R^2 = 0.207***, P <0.001). These results suggest that both root hair related mutants (genotypes) and drought stress (treatments) jointly shape root-associated microbiome changes upon drought stress.

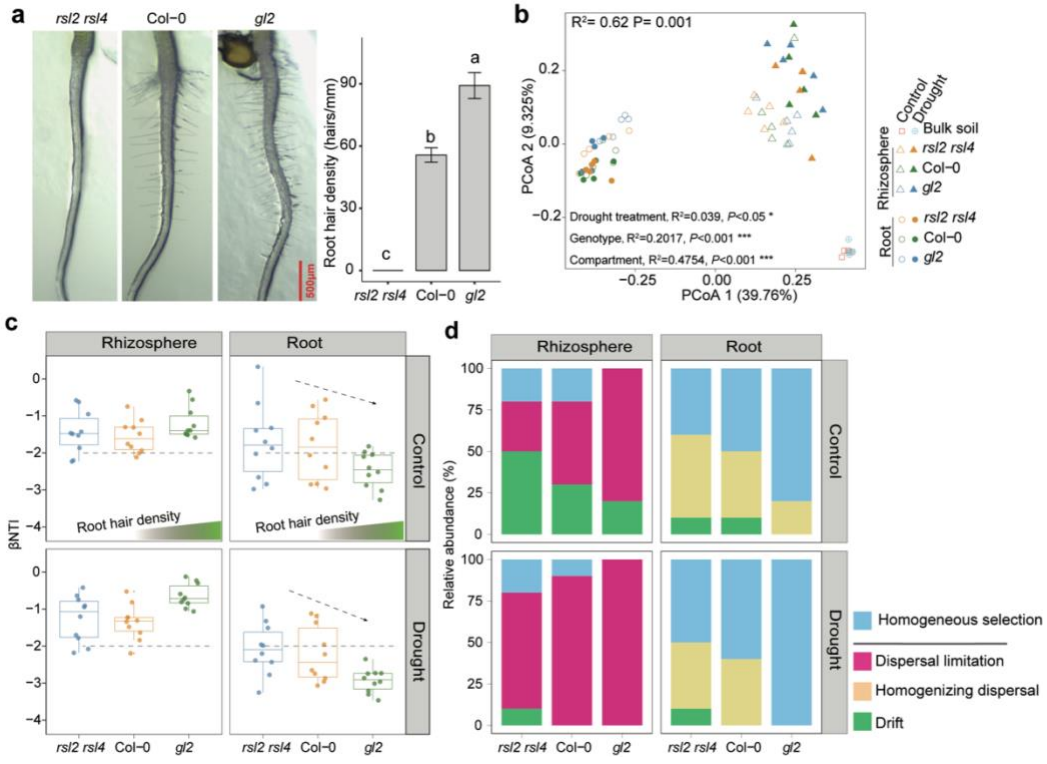

Fig. 1 Root hair mutants shift microbiome composition under drought stress. a Photos and quantification of root hair densities of the 5-day-old roots of Col-0, rsl2 rsl4 and gl2. Data are represented as mean (bar) ± standard error of mean (error bar). Experiment was repeated twice with consistent results. Different letters represent the significant ($p < 0.05$ corrected using Bonferroni method, one-way ANOVA followed by LSD test) differences among genotypes (N=40 biological replicates). b. Principal coordinates analysis based on Bray–Curtis dissimilarity that calculated from the relative abundance matrices at ASV level (PERMANOVA by adonis, n=5 replicates for each individual group). c The β-nearest taxon indexes (βNTI) in different genotypes. Each dot represents the β-nearest taxon index calculated from each pairwise sample in each genotype and treatment condition. The dotted line represents the cutoff for determining deterministic (|βNTI|>2) and stochastic (|βNTI|<2) processes. Box plots show the median (horizontal bar), 25th (bottoms of boxes) and 75th (tops of boxes) quartiles range (QR), and non-outlier

data value (upper and lower whiskers) of β-nearest taxon index value in each group. d Percentage of relative influence of each community assembly cues were defined as proportion of pairwise samples governed by each process. The horizontal line in the legend represents the boundary between the main ecological processes driven by deterministic processes (above) and stochastic processes (below).

gl2 mutant drives a deterministic microbiome assembly process during drought stress

Understanding the assembly cues of microbial communities helps us understand the factors that influence community changes[38]. To assess whether plant root hair related genetic effects contribute to a deterministic process in microbiome assembly, we calculated the β-nearest taxon index (βNTI) indexes in the microbiome samples of root hair related mutants (|βNTI| >2 indicates a deterministic process[38]). Forn rhizosphere samples, a stochastic process (-2<βNTI<0) governed the assembly of genotype-specific bacterial communities in both the control and drought-treated groups (Fig. 1c), suggesting a relatively weak rhizosphere effect in regulating rhizosphere microbiome changes. In contrast, we detected a stronger effect of genotype on root microbiome assembly. With the increase in root hair density, the root microbiome assembly shifted from a co-governed pattern by stochastic and deterministic processes (in roots of *rsl2 rsl4* and Col-0) to a pattern primarily governed by deterministic process (in roots of *gl2*). Importantly, the assembly of bacterial communities in the roots of *gl2* was completely governed by a deterministic process (βNTI < -2) under drought conditions(Fig. 1c). That means enhanced root hair density has strong deterministic influence on drought induced root microbiome changes.

We further calculated the Bray-Curtis-based Raup-Crick index (RCbray) to quantify the contribution of different community assembly cues. This index allowed us to assess the proportion of pairwise community comparisons dominated by each process. In rhizosphere samples, dispersal limitation (βNTI < -2 and RCbray > 0.95) governed the stochastic process in all genotypes under drought (Fig. 1d). Interestingly, for root samples, the deterministic process were dominated by homogenous selection(Fig. 1d; βNTI < -2). Importantly, the relative influence of homogenous selection in the assembly of bacterial communities was much higher (75% - 100%) in *gl2* than in *rsl2 rsl4* (below 50%) and Col-0 (approximately 50%). Our results provided genetic evidence about the involvement of plant effects in deterministic microbiome assembly under drought stress.

Rhizobiaceae are the key taxa regulated by root hairs under drought stress

To further explore the key taxa influenced by root hairs under drought conditions, we analyzed the relative abundance of different taxonomic levels among genotypes. Mutants exhibited clear shifts at the family level (Supplementary Fig. 1d). We found that the abundance of *Rhizobiaceae* was significantly higher in *gl2* than *rsl2 rsl4* under drought stress (Supplementary Fig. 2b). Notably, by using a RandomForest predicting[39], we identified *Rhizobiaceae* as the most effective family in distinguishing the three genotypes (Fig. 2a), and also with the highest abundance among all biomarker families identified (Fig. 2b).

We further performed differential abundant analysis of ASVs between individual mutants and Col-0. We found that the differential ASVs (DA-ASVs) from rhizosphere samples between the two root hair mutants and Col-0 were distributed across multiple families, regardless of whether it was in the drought or control group (Supplementary Fig. 2a-d). However, for DA-ASVs in root samples were enriched in a few families, especially under drought condition (Supplementary Fig. 2e, f; Fig. 2c, d). We detected 5 significantly enriched ASVs in *gl2*, and 3 of them belong to *Rhizobiaceae* (Fig. 2c). Only 1 ASV (belonging to *Comamonadaceae*) was enriched in the root microbiome of *rsl2 rsl4* (Fig. 2d). Collectively, our machine learning (RandomForest) based biomarker prediction, as well as differential abundance analysis at the family level, all support that *Rhizobiaceae* are major biomarker taxa regulated by root hairs under drought stress.

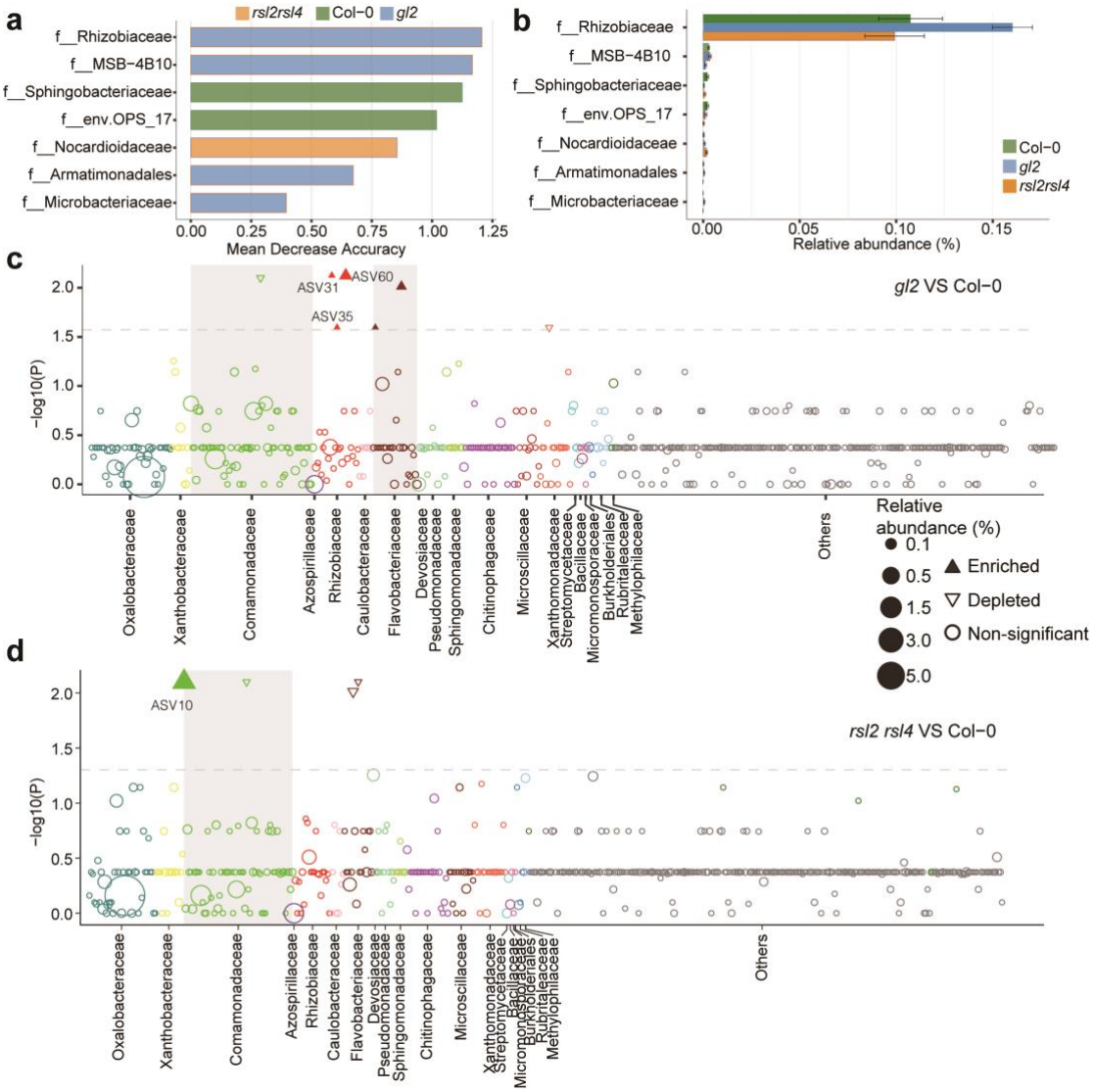

Fig. 2 Biomarker taxa and species between mutants and Col-0. a-b A total of 7 marker families were identified under drought conditions by Kruskal-Wallis rank sum test and random forest classification (a) and their corresponding relative abundances (b) in each genotype. In bar plot, data are represented as mean (bar) ± standard error of mean (error bar). c-d Manhattan plot showing the differential abundance ASVs enriched or depleted in the

roots of gl2 (c) and rsl2 rsl4 (d) compared to Col-0 (Wilcoxon rank sum test, unadjusted $p < 0.05$) under drought condition. n=5 biological replicates for microbiome analysis in each genotype and treatment conditions. Each dot and triangle represent each ASV. ASVs were colored according to the taxonomic family. Size of each dot or triangle represent the relative abundance of each ASV. Solid upward triangles indicate the ASV s enriched in the roots of mutant. Hollow downward triangles represent the ASVs depleted in the mutants.

gl2 mutant affects the network importance of *Rhizobiaceae* nodes

Complex microbe-microbe interactions substantially affect network structure and stability, which further affect community function[40]. Network analysis is widely used to identify keystone taxa that play important roles in shaping microbial community structure and affecting community functions[41,42]. Considering that we only observed a deterministic assembly process in root but not rhizosphere microbiome (Fig. 1c, d), we subsequently focused on the network interactions within root microbiome. To further explore whether and how root hairs affect network interactions in root microbiomes, we constructed the co-occurrence networks based on Spearman's correlations (with a correlation coefficient threshold of 0.7, $P_{FDR}<0.05$) between paired ASVs in the root microbial communities within each genotype. We found that, compared to Col-0 (Fig. 3a, nodes under control=89; Fig. 3d, nodes under drought=118), the hairy mutant *gl2* has much larger (total number of nodes) and more complex microbiome networks under both control (Fig. 3c; nodes=287) and drought (Fig. 3f; nodes=147) conditions. Additionally, the network connectivity (total number of edges) and average connectivity (average links/degree per nodes) are also higher in the *gl2* mutant compared to Col-0 and *rsl2 rsl4* (Fig. 3a-f; Supplementary Table 3). By contrast, there was no significant difference in network size and connectivity between the co-occurrence networks of *rsl2 rsl4* and Col-0 (Fig. 3a, b, d, e). Our data strongly supports that the increase in root hair density leads to increased complexity in the networks of root microbiomes, both under control and drought conditions.

Differences in the network topological properties of different nodes (ASVs) determine the importance of each node in the network. Degree centrality (the number of edges connected to the node) and closeness centrality (average length of the shortest path between the node and all other nodes) are broadly used to describe the importance of network nodes. We observed that ASVs belonging to the top six families (top 10 abundance-ranked and the top 10 network-node-count-ranked overlapping families from all genotypes) exhibited significantly higher mean degree centrality in the network of *gl2* under control, compared to Col-0 and *rsl2 rsl4* (Fig. 3g; ANOVA analysis followed by Fisher's LSD test, $P < 0.05$ corrected using Bonferroni method). The ASVs belonging to *Rhizobiaceae*, *Comamonadaceae*, and *Oxalobacteraceae* showed significant differences in mean degree centrality in *gl2* compared to Col-0 under drought conditions. The mean degree centrality of the ASVs belonging to *Rhizobiaceae* consistently increased from *rsl2 rsl4*, Col-0 to *gl2*, indicating a crucial role of root hairs in maintaining the network importance of *Rhizobiaceae* nodes (Fig. 3h).

Hub nodes (ASVs) were usually identified based on their degree centrality, closeness centrality,

and betweenness centrality indexes[43]. We further analyzed all those indexes related centrality, and two hub ASVs were identified in the network of *gl2* under drought treatment, and both are belonging to *Rhizobiaceae* (Supplementary Fig. 3). We also detected increased interactions with *Rhizobiaceae* nodes in *gl2* under drought conditions. In the co-occurrence network of *gl2*, the edges interacting with the ASVs belonging to *Rhizobiaceae* increased from 16.2% to 24.7% from control to drought condition (Supplementary Table 3). Collectively, our results suggest that increased root hair density enhances network complexity and importance of ASVs belonging to *Rhizobiaceae* in the root microbiome.

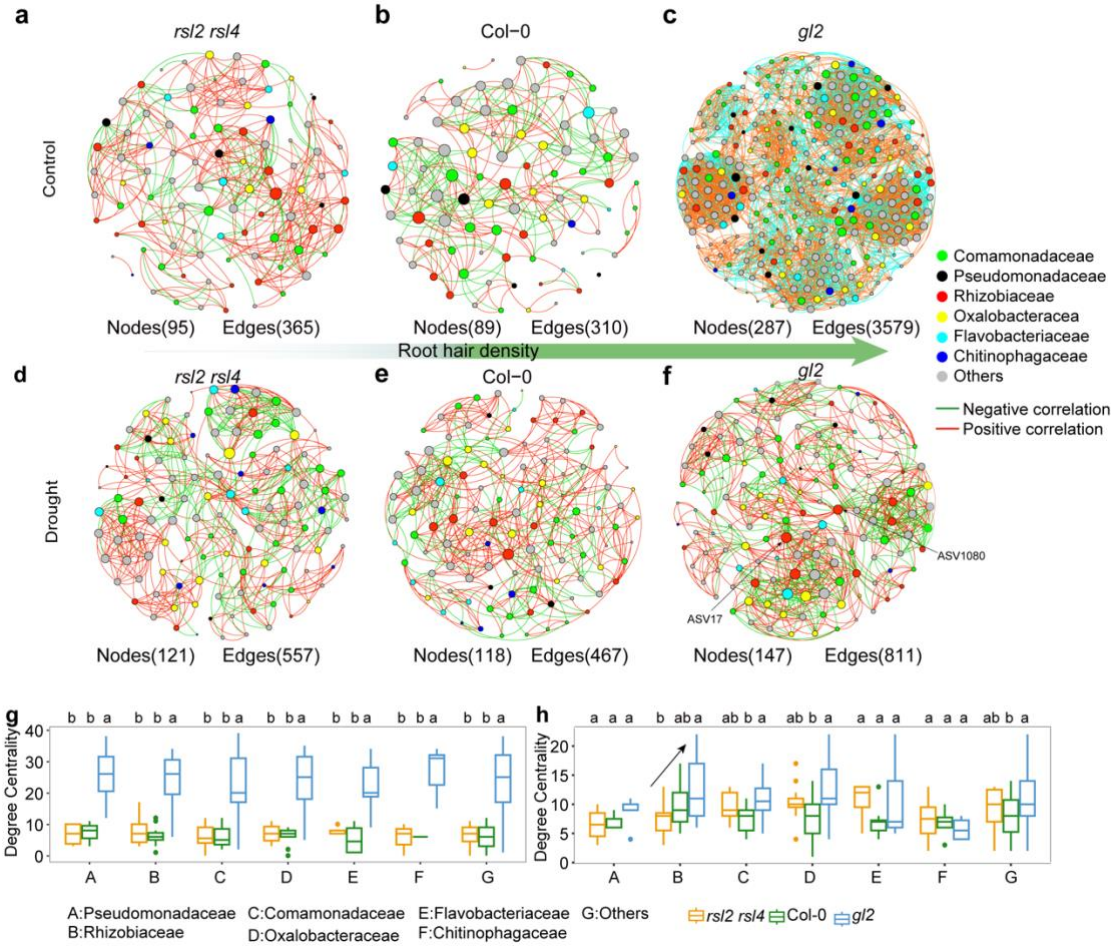

Fig. 3 Changes in the microbial co-occurrence networks of root-associated samples of root hair mutants and Col-0. a-c Co-occurrence networks of microbiomes in the root samples of rsl2 rsl4 (a), Col-0 (b), gl2 (c) root samples under control. Red dots indicate nodes belonging to Rhizobiaceae. d-f Co-occurrence networks of microbiomes in the rhizosphere samples of rsl2 rsl4 (d), Col-0 (e), gl2 (f) under drought condition. The exact numbers of nodes and edges were indicated below each graph. The arrow indicated the hub nodes within each network. g-h The distributions of degree centrality of ASVs from the top 6 families across microbiome networks in the roots of different genotypes under control (g) and drought conditions (h). The different letters represent the significant ($p < 0.05$ corrected using Bonferroni method, one-way ANOVA followed by LSD test) differences among genotypes. Box plots show the median (horizontal bar), 25th (bottoms of boxes) and 75th (tops of boxes) quartiles range (QR), and non-outlier data value (upper and lower whiskers) of ASV's degree centrality within each family.

gl2 mutant broadly shifts the composition of root metabolome and enriches flavonoids

Root exudates play a pivotal role in shaping genotype- or stress-specific microbiomes[44,45]. To investigate the potential metabolic cues related to *gl2gl2* mediated microbiome re-shaping under drought stress, we conducted non-targeted metabolomics analysis of chemical compositions in the roots of *gl2*, *rsl2 rsl4*, and Col-0. A total of 3920 compounds were identified in root samples from different genotypes. Principal component analysis (PCA) revealed significant differences in the biochemical composition of root metabolites (R^2= 0.58, P<0.05, PERMANOVA by adonis) among these genotypes (Fig. 4a). The content of flavonoids is significantly higher (7.1%) in the roots of *gl2* compared to that in Col-0 (4.5%) and *rsl2 rsl4* (2.1%) (Fig. 4b). Conversely, the relative content of ketones was higher in the roots of *rsl2 rsl4* mutant (51.4%) compared to Col-0 (32.6%), but lower in the roots of *gl2* mutant (11.7%) (Fig. 4b). Indole and its derivatives, as well as organic acid and its derivatives, were higher in the root of *gl2* compared to Col-0, but lower in *rsl2 rsl4*. The metabolome profiling results suggest that mutations in root hair development can significantly alter the composition of metabolites in roots under drought.

Subsequently, differentially abundant metabolites (DMs) were identified based on variable importance in projection scores (VIP > 1.0) and fold changes (logFC > 0) of the relative abundances (P<0.05). A total of 383 DMs were identified between *gl2* and Col-0 (Supplementary Table 4), and 964 DMs were identified between *rsl2 rsl4* and Col-0 (Supplementary Table 5). Enrichment analysis was further performed based on DMs in *rsl2 rsl4* and *gl2*, respectively. Consistent with the increase in relative content of overall flavonoids, the DMs in the roots of *gl2* are enriched in pathways associated with flavonoid biosynthesis, as well as pathways related to caffeine metabolism, purine metabolism, and nucleotide metabolism (Fig. 4c). In contrast, the DMs in *rsl2 rsl4* are mainly enriched in pathways related to C5-Branched dibasic acid metabolism, plant hormone signal transduction, butanoate metabolism, and monobactam biosynthesis (Supplementary Fig. 4a). A previous study showed that flavonoids accumulation in roots can induce the chemotaxis of *Aeromonas* and enhance plant dehydration resistance[46]. Furthermore, flavonoids (e.g., naringenin) are well-known for their effects on inducing the expression of rhizobia nod genes in leguminous plants, as well as the chemoattraction of rhizobia towards the roots[47]. In the present study, tangeretin (a flavonoid molecule) in the roots of *gl2* exhibited the highest VIP value (Supplementary Table 3; P < 0.001) among the DMs with a more than 3 folds enrichment compared to Col-0 (Fig. 4d, Supplementary Fig. 4b). The above results comprehensively revealed the metabolome changes in *gl2* during drought stress, including the enrichment of flavonoid compounds which were reported to be related to rhizobia colonization in leguminous plants.

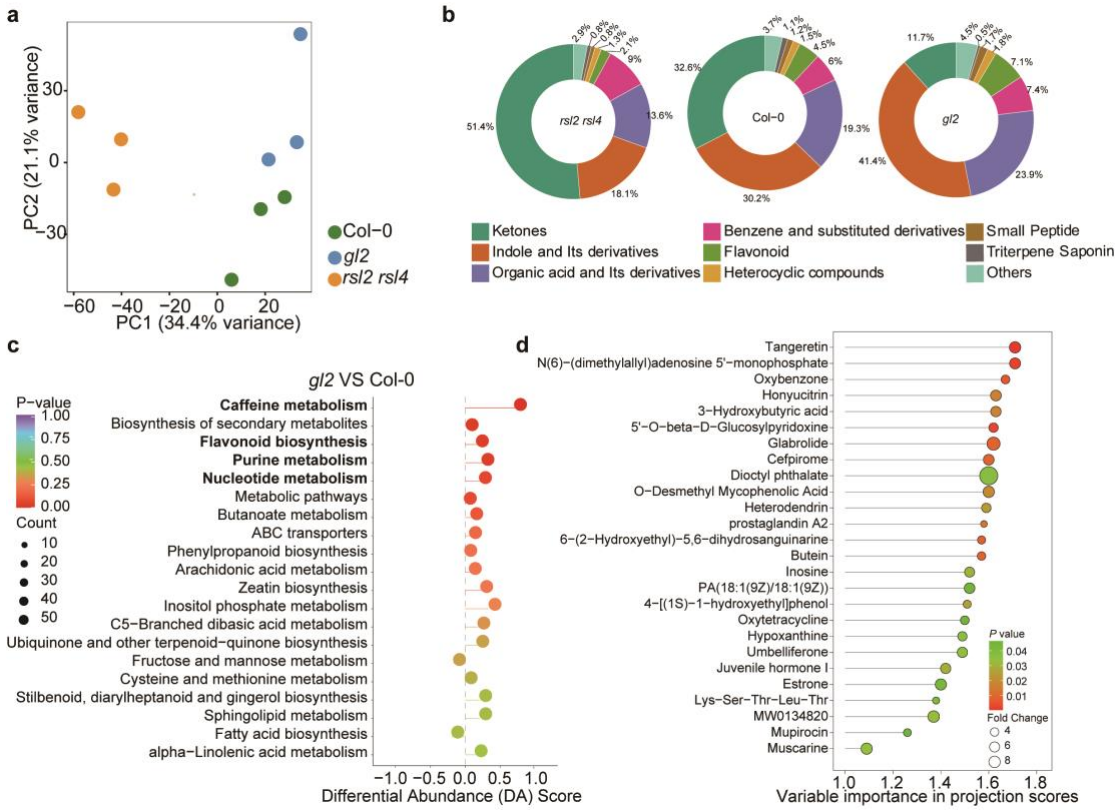

Fig. 4 Metabolomic profiling of root samples from different genotypes under drought stress. a Principal component analysis (PCA) of metabolites detected in the roots of Col-0, *gl2* and *rsl2 rsl4* ($p < 0.001$, permutational multivariate analysis of variance (PERMANOVA) by Adonis). N=3 biological replicates. **b** Relative content of different metabolites classes in the roots of Col-0, *gl2* and *rsl2 rsl4*. **c** Enriched pathways of differentially abundant metabolites (DMs) in *gl2*compared to Col-0 under drought condition. The differential abundance (DA) score represents the number of changed DMs relative to the total numbers of metabolites within each pathway. Colors indicate p-values and size of each dot indicate the number of metabolites. **d** Variable importance of projection (VIP) scores of all the DMs (fold change > 2 are displayed) in the roots of *gl2*. The colors indicate p-value. The size of each circle represents the fold changes of relative contents in *gl2* relative to Col-0.

Discussion

In the era of the host-microbiome holobiont, understanding how host genetics facilitate the recruitment of beneficial microbes under stresses is a fundamental question critical for engineering plant microbiome. To address this, we need to integrate genetic approaches, multiple-omics and reductionist based confirmations. Thanks to the power and low cost of plant genetics approaches, previous studies have been well established that plant genetic effects positively contributes to recruiting disease suppressive microbes[8,19,48]. In contrast, while drought is the most serious abiotic stress which drastically disturbs root associated microbiome, whether and how plant genetic effects positively recruit stress-alleviating microbes during drought is largely unknown. By taking advantage of elaborately selected genetic mutants with a gradient of root hair densities, we were able to confirm the crucial role of plant root hairs in positively re-shaping drought triggered microbiome changes. Our microbiome composition and network analyses revealed that *Rhizobiacea*

are the major family regulated by root hairs. Our work supports the essential roles of plant genetic effects in positively re-shaping a drought alleviating microbiome, and furthers our understanding about the “cry for help” phenomenon during abiotic stress.

Similar to gut microvilli, plant root hairs substantially increase root surface area and play essential roles in nutrient and water uptake. Conventional crop breeding programs typically prioritize high root hair density and length as desirable traits in agriculture, primarily from a nutrient uptake perspective[49,50]. However, our work reveal a critical novel role of root hairs in orchestrating the beneficial interactions with root associated microbiomes. This is reminiscent of a recent study which surveyed the root cell type specific transcriptome responses to beneficial *Pseudomonas simiae* WCS417 in roots, and found that root hairs show special immune responsiveness to WCS417[51]. Root hair related mutants also dampen the immune responses and compatible interactions with WCS417, highlighting the crucial roles of root hairs in sensing and regulating the interactions with beneficial microbes[51]. Furthermore, our recent single-nucleus RNA-seq analysis discovered that beneficial microbes (*Pseudomonas simiae* WCS417) and pathogenic microbes (*Ralstonia solanacearum* GMI1000) trigger very distinct (only 11.84% overlap) transcriptome responses in root hairs (trichoblasts)[52]. Beneficial WCS417 promotes the expression of growth-related GO terms related to ribosome functions, while the pathogenic GMI1000 triggers senescence and phosphorelay signaling-related stress responses in root hairs[52]. This further supports the special role of root hairs in differential responses to beneficial and pathogenic microbes in roots. Since both root hairs and gut microvilli are continually exposed to extremely diverse microbiomes, these cell types might share similar microbial ecological roles in mediating host-microbiome interactions. Further molecular and microbial ecological studies in the plant system would advance our understanding of the rules governing host-microbiome interactions in these "frontline" hair cells.

Although *Rhizobiaceae* is one of the most well-studied plant symbiotic bacterial families in leguminous plants, our work suggests that they confer fitness benefits and act as microbiome network hubs in the non-leguminous plant *Arabidopsis*. A previous study surveyed the core microbiome compositions across diverse plant lineages, from non-seed to seed plants (31 plant species), and characterized Bradyrhizobium and Rhizobium as universally plant-enriched core microbiome taxa[53]. Phylogenetic analysis among 1,314 Rhizobiales genomes suggested that both nodulating and non-nodulating strains share common genes related to root colonization[54]. The evolutionarily conserved associations between *Rhizobiaceae* and roots indicate their crucial roles in the structure and function of root-associated microbiomes. Moreover, a previous study in Medicago suggests that *Rhizobiaceae* are critical hub species in the root microbiome, and that genetic mutants that loss association with *Rhizobiales* also show altered microbiome structure[55]. Interestingly, in our non-leguminous *Arabidopsis*, we also detected that enhanced root hair density is associated with enhanced colonization levels and more edges connected to *Rhizobiaceae* nodes in the microbial co-abundance network, especially under drought stress. This further suggests the potentially conserved roles of *Rhizobiaceae* in mediating microbe-microbe interactions in root microbiome network.

Most mechanistic studies about root-*Rhizobiaceae* interactions were conducted in legume plants[56]. *Rhizobiaceae* can not only enter root tissues in leguminous roots but also in non-leguminous plants like rice[57]. This suggests that *Rhizobiaceae* could be endophytes for both leguminous and non-

leguminous plants. A recent study systematically surveyed the transcriptome responses to *Rhizobiaceae* in roots, and found that NAC060 is a key regulator of Rhizobiales specific responses[58]. More importantly, they identified that host sulfated peptide (phytosulfokine) signaling is essential for *Rhizobiaceae* mediated growth promoting effects, providing critical insights about the molecular mechanisms underlying root-Rhizobiales interactions[58]. Considering the powerful genetic and genomic tools in *Arabidopsis* and *Rhizobiaceae*, this interaction system would likely facilitate new discoveries about root-commensal interactions.

Material and methods

Plant material and growth conditions

All the seeds were surface sterilized for 20 minutes with chlorine gas (exposure to 100ml bleach plus 5ml concentrated hydrochloric) to eliminates potential endophytes[59]. Sterilized seeds were soaked in a 0.1% agar solution and stored at 4℃ in the dark for 2 days before use. Seeds were germinated on 1/2x Murashige and Skoog (MS) agar plates with 1% sucrose (12 h light/12 h dark). 7-days-old seedlings were transplanted into pots filled with soil (as described below) in greenhouse. Plants were grown under a 10 h light (light intensity: 100 μmol m^{-2} s^{-1})/14 h dark condition at 22℃.

Natural soil gorwth substrates

The natural soil used in this study were collected separately from Yuanjiang Savanna Ecosystem Research Station (E102° 10’,N23° 28’) and Xishuangbanna Tropical Botanic Garden (E101° 27′,N21° 92′) of Chinese Academy of Sciences. Visible stones, plant debris and litter were removed before harvesting natural soil. Natural soil was sieved through a 2 mm sieve. Soil from different locations were thoroughly homogenized and then mixed in a 1:1 ratio as a mixed natural soil. Finaly, we set up a mixture substrate composed of equal volumes (1:1:1:1) of mixed natural soil, commercial growth roon soil, vermiculite, and perlite as soil substrate for all natural soil experiments in this study. The soil were scooped into 6cm by 6cm pots in the greenhouse for plants transplantation. To sterilize natural soil, we autoclaved the substrate twice (at 121℃ for 20 minutes each time) with at least 24 hours interval between two auto-claving (which thoroughly kills potential germinated microbe spores after the first time autoclaving). We added fertilizer once per week for natural soil growth substrates.

Drought treatments

Plants were transplanted from plates into natural soil growth substrates at 7 days after germination. After normal watering for three weeks, plants within each genotype were randomly assigned to drought and control treatments. We conducted drought treatment by totally withholding watering and randomly rotating all plants every day.

Sample collection

We scooped whole plant outside the pot and removed the soil that was not closely adhered by

shaking the roots[60]. The roots tissue of four plants were immediately cut below the shoot-root junction in a 50 ml Falcon tube filled with 25 ml of sterile PBS (10mM/L). After shaking the Falcon tube for 20 minutes at 180 rpm, the roots were transferred to a new 10 ml Falcon tube filled with fresh PBS. The washing buffer was centrifuged for 20 min at 4000g (16 ℃) and the resulting pellet was defined as rhizosphere sample. For root samples collection, roots in fresh PBS were thoroughly washed and sonicated (at 40 Hz for 30s) twice to further discard remaining soil. The pot soil without plant and after removing 2cm of top soil was defined as bulk soil. All the samples were transferred into a new 2 ml tube, immediately frozen in liquid nitrogen, and then stored at -80 ℃ before DNA extraction.

DNA extraction and microbiome sequencing

DNA extraction was performed using PowerSoil DNA Isolation Kit (Qiagen, Germany) following the manufacturer's protocol. The DNA samples that concentration higher than 20 ng/ μl were used for microbiome sequencing. For bacteria amplicon sequencing, the V3-V4 region of the 16s rRNA gene was amplified with primers 349F 5'-ACTCCTACGGGAGGCAGCA-3' and 806R (5'-GGACTACHVGGGTWTCTAAT-3'. Amplification was carried out following thermal conditions: 94℃ for 5min, followed by 30 cycles of 94℃ for 30 seconds, 52℃ for 30 seconds, and 72℃ for 30 seconds, with a final extension at 72℃ for 10 minutes. Library was prepared following the protocal of the NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (New England Biolabs,USA). The library concentration were determined by Qubit 4.0 Fluorometer. For amplicon sequencing, paired-end 250 bp sequencing was performed on Illumina Novaseq 6000.

Microbiome data processing

Raw sequencing reads were filtered using fastp v.0.14.1[61]. The adapter sequences and primers were further removed using cutadapt v.4.0[62]. Sequencing reads were processed using QIIME2 v.2022.2 [63]. Briefly, DADA2 was used to generate a table of unique amplicon sequence variants (ASV) and their counts per sample [35]. For taxonomic annotation, representative sequences of each ASV were assigned to the SILVA database (release 138) using pre-trained naive Bayes classifier [64,65]. Unassigned sequences and that annotated as chloroplast and mitochondria (considered as host contamination) were removed. In addition, the ASV that present in less than 3 samples were also removed. The retained ASVs were used for downstream analysis.

Microbial community assembly processes

The null model analysis was carried out to evaluate the microbial community assembly process by calculating the β-nearest taxon index (βNTI)[38]. Firstly, we calculated the observed abundance-weighted β-mean-nearest taxon distance ($\beta MNTD_{obs}$) of pairwise community (sample) with *comdistnt* function in the "picante" package in R[66]. By randomly shuffling the tips of the phylogenetic tree, the null model expectation and distribution of βMNTD ($\beta MNTD_{null}$) were generated through 999 times randomization. The βNTI were then calculated to quantified the

standard deviations that the $\beta MNTD_{obs}$ from the distribution of $\beta MNTD_{null}$ of pairwise community. When $|\beta NTI| > 2$, it is interpreted as the community assembly being governed by deterministic process. Conversely, when $|\beta NTI| < 2$ indicated that community assembly dorminated by stochastic process. Heterogneous seection and homogeneous selection in deterministic process were repectively estimated with a value of $\beta NTI > 2$ and $\beta NTI < -2$[67].

To further assess the contributions of stochastic and deterministic process ($|\beta NTI| < 2$) in community assembly process, a previously developed Raup-Crick index (RC_{bray}) were calculated as described previously [68]. When pairwise community comparison with $|RC_{bray}| > 0.95$, indicated that dispersal lead to a community turnover. When $RC_{bray} > 0.95$, the community turnover was dominated by dispersal limitation, whereas homogenizing dispersal when RCbray < -0.95. When pairwise community comparison with $|\beta NTI| < 2$ and $|RC_{bray}| < 0.95$, community turnover is estimated to be affected by drift alone. Collectively, the value of βNTI and RC_{bray} were integrated to assess the deterministic and stochastic process in microbial community assembly, as well as relative influence of the each specific process.

Metabolome profiling

For sample collection, fresh roots of 16 plants grown in the natural soil were harvested as one biological replicate. Root samples collection steps are same as harvesting root samples for microbiome sequencing. After sampling, root samples were quickly (within 5 minutes) and thoroughly washed in 200 ml sterile deionized water. Then root tissue was immediately frozen in the liquid nitrogen. Tissue fresh weight was recorded and then stored at -80 ℃ until further processing.

Non-targeted metabolomics were performed to investigate the impact of root hair mutation on root metabolites under drought condition. Metabolites detection and identification were conducted using a ultra-performance liquid chromatography (LC-30A, Shimadzu, Japan)–tandem mass spectrometry (TripleTOF 6600+, SCIEX) system (UPLC-MS/MS) in Wuhan Metware Biotechnology Co., Ltd. (Wuhan, China) (http:// www. metwa re. cn/).

For metabolome data processing, principal component analysis (PCA), which implemented by function prcomp in the “stats” package in R (www.r-project.org), was used to assess differences in root metabolites among root hair mutants and Col-0. Differential abundance metabolites (DMs) between each mutant and Col-0 were determined by variable importance in projection (VIP>1), *P* value (*P* value < 0.05, Student’s t test) and fold change ($|log2FC| > 0$) differences. VIP values were extracted from Orthogonal Partial Least Squares-Discriminant Analysis (OPLS-DA) result, which was generated using R package MetaboAnalystR[69]. The heatmap showing the relative abundance of differential metabolites between mutant and Col-0 were generated by using the “ComplexHeatmap” package in R[70]. Differential metabolites between mutant and Col-0 were annotated using KEGG compound database (http://www.kegg.jp/kegg/ compound/), and further mapped to KEGG Pathway database (http://www.kegg.jp/kegg/ pathway.html).

Statistics analysis and data visualization

All statistics analyses in present study were conducted in R v.4.1.3 environment (http://www.r-project.org/). Before different statistics analysis were chosen, normality tests were performed using the Shapiro-Wilk test. Then Bartlett's test was used to test for homogeneity of variances using "stats" package in R. The "vegan" package in R was utilized to assess of alpha and beta diversity of root-associated microbial community[71]. Specifically, species richness, Shannon diversity were calculated with *diversity* function. The differences between root hair mutants and Col-0 were assessed using a one-way ANOVA, followed by an LSD test for multiple comparisons. This analysis was performed using LSD.test function in "agricolae" package in R[72]. Bray–Curtis distance matrices of microbial communities were calculated using the *vegdist* function, and principal coordinate analysis (PCoA) plots were generated accordingly**.** Permutational multivariate analysis of variance (PERMANOVA) was performed with the "*adonis*" function to further assess effects of genotype (Col-0, *gl2* and *rsl2 rsl4*), water regime (drought and control), compartment (bulk soil, root and rhizosphere) on variation of microbiome composition[71]. The "randomForest" and "microeco" packages in R were utilized to perform differential abundance tests and identify potential marker families among three genotypes[39,73]. Wilcoxon rank sum test were used to detect the differential (FDR adjust $p<0.05$) ASVs (based on relative abundance) between each root hair mutant and Col-0.

For network analysis, the ASVs with a relative abundance greater than 0.01% and present in at least two samples were used for co-occurrence network construction. Spearman correlation (correlation coefficient >0.7 , $P_{FDR}<0.05$) analysis was conducted between paired ASVs using the "ggClusterNet" packages in R[74]. Network topological parameters, including node and edge counts, positive and negative correlations, as well as node properties such as degree centrality, closeness centrality betweenness centrality, were calculated using the "igraph" package in R[75]. Hub nodes were identified based on all these three measurements of centrality using a log-normal distribution fit[43]. With the exception of the network visualization, which was conducted in Gephi 0.10[76], all the plots in present study were generated using "ggplot2" package in R[77].

Data availability.

All raw 16S amplicon and shortgun metagenoimic sequencing data reported in present study will be deposited in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra) upon publication.

Author contributions

Y.S. designed the project, Z.W. and Z.L. conducted experiments with the help from J. Z., X. T. and K. G. Z.W. analyzed data. Y.S and Z.W. wrote the manuscript.

Acknowledgements

We thank Drs. Hongwei Guo and Yichuan Wang for sharing *gl2* and *rsl2 rsl4* mutants. We are grateful for Dr. Yajun Chen, Drs.Wenjun Zhou (Xishuangbanna Tropical Botanic Garden(XTBG), Chinese academy of science), Guojing Wen, and Daxin Yang (Yuanjiang Savanna Ecosystem

Research Station, XTBG) for the collection of nature soil. The project was supported by the Stable Support Plan Program of Shenzhen Natural Science Fund Grant (20220815160107001) and the NSFC General Project (32270286).

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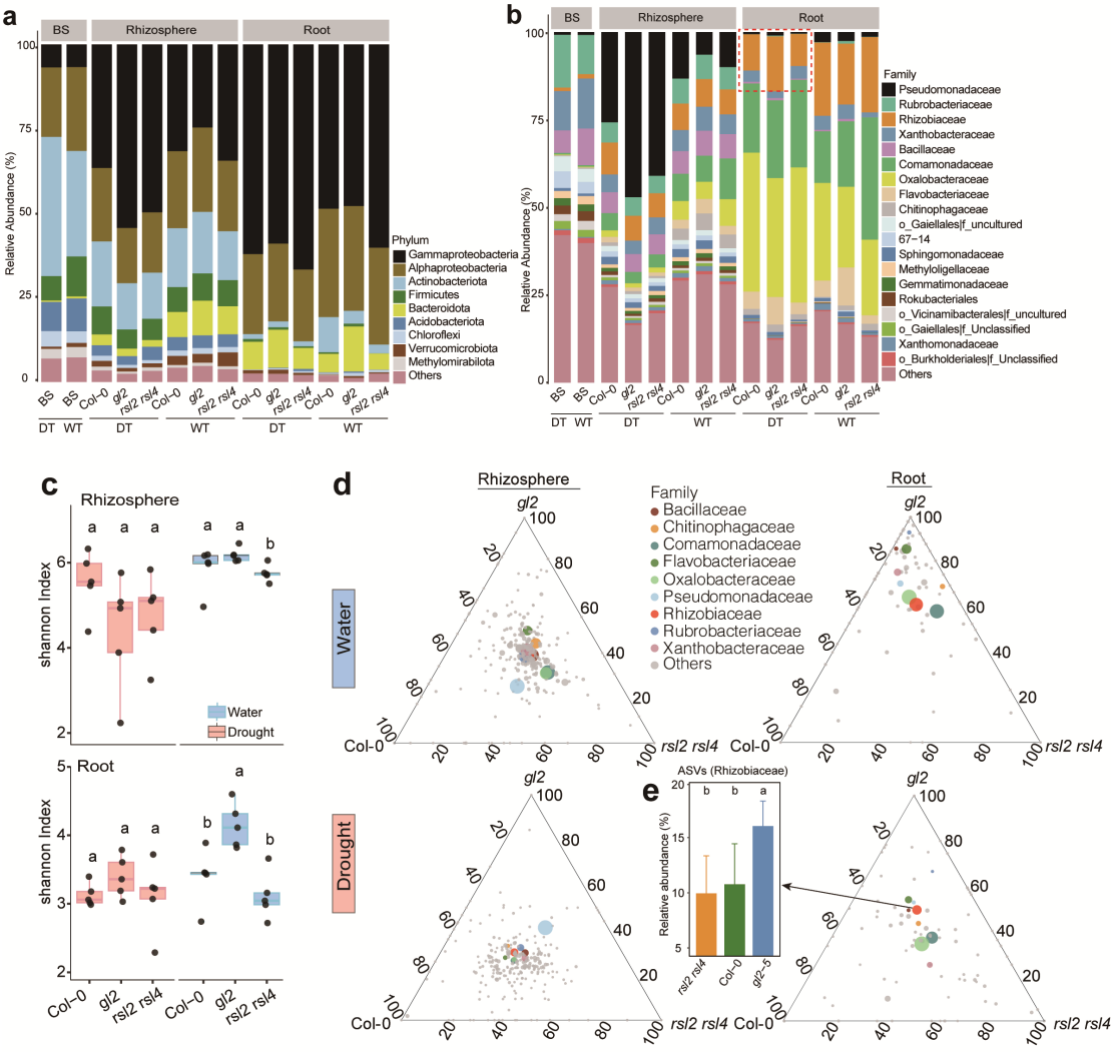
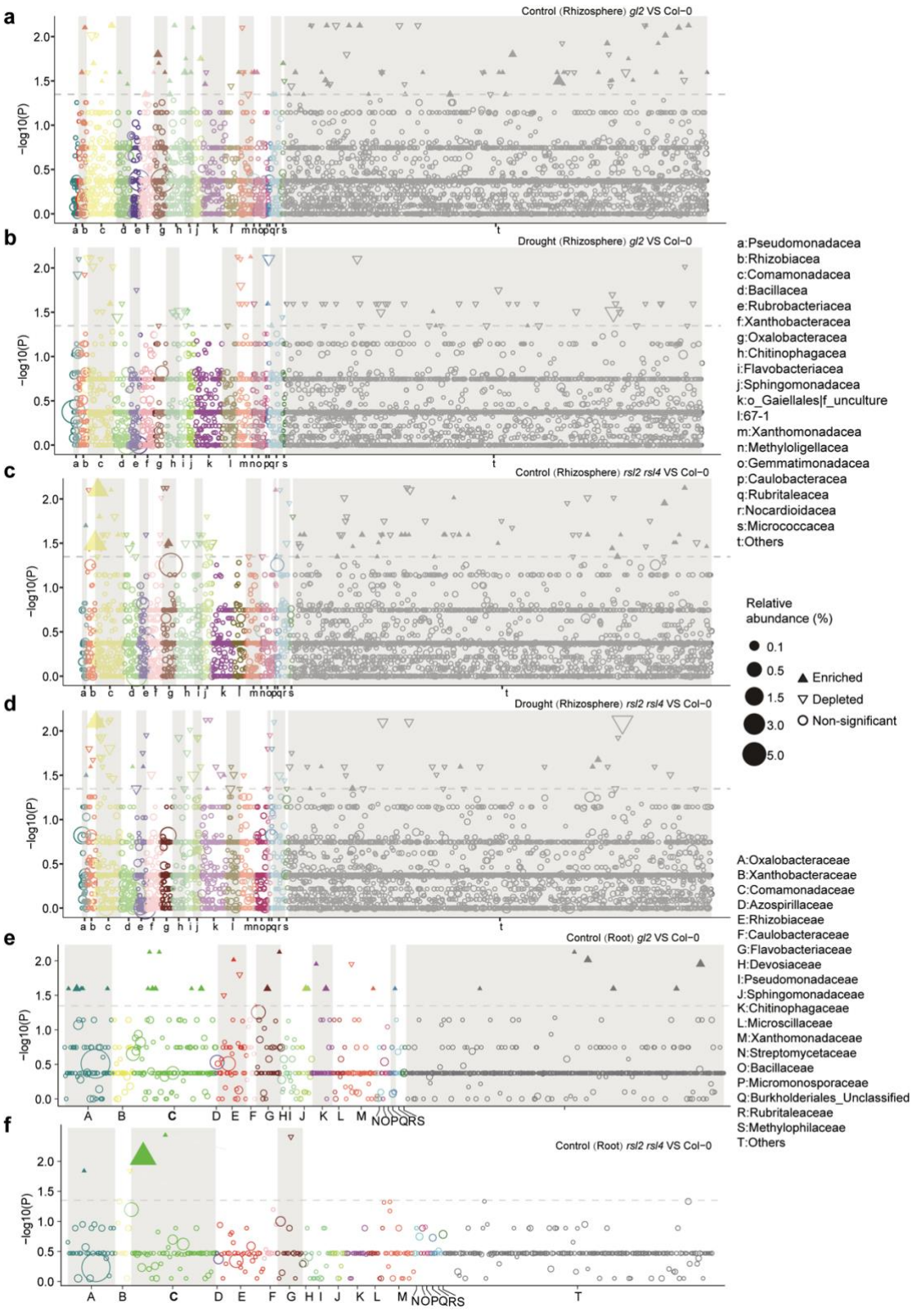
Supplementary Figure



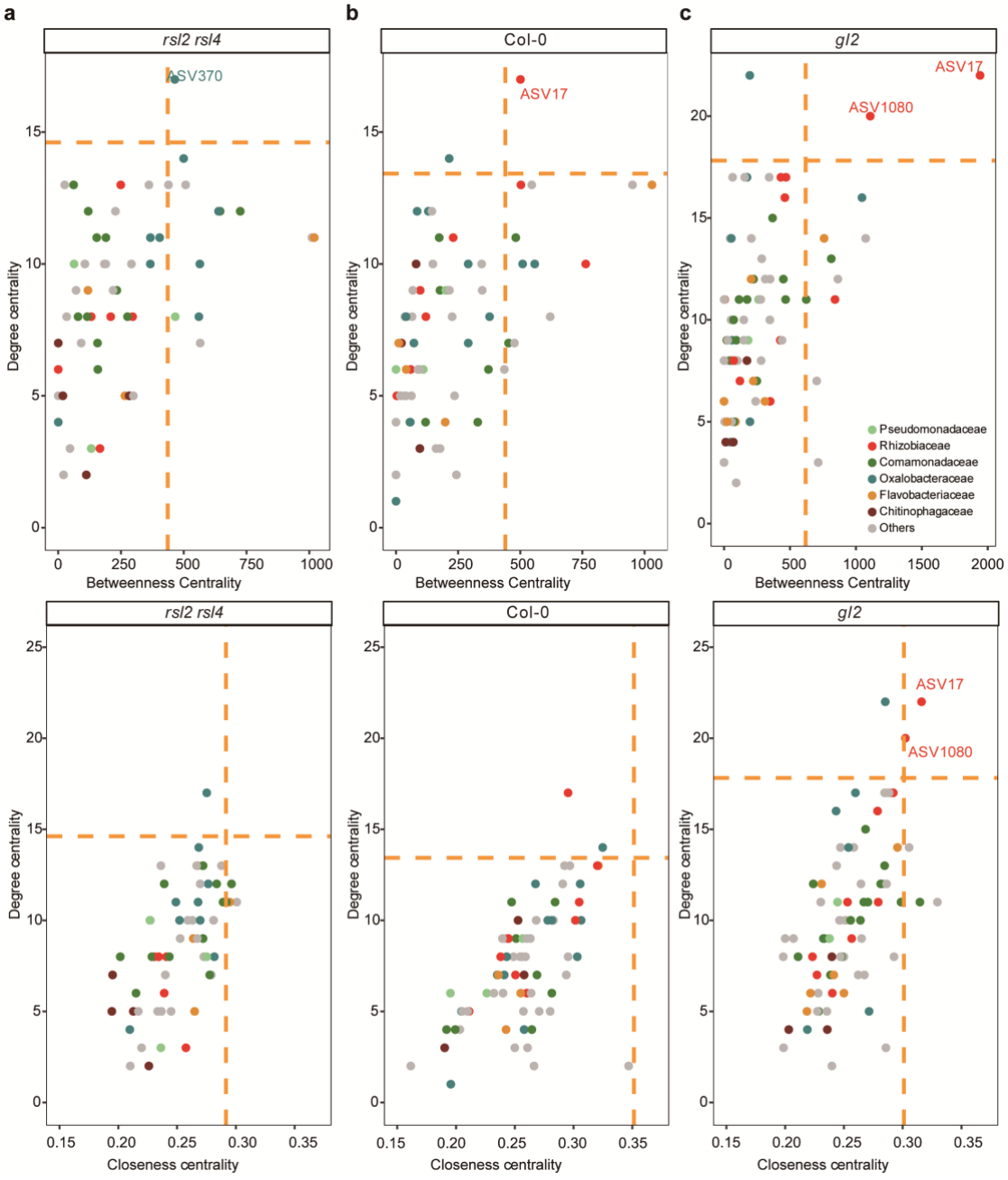
Supplementary Fig. 1. a-b Relative abundance of phyla (a) and family (b) level compositions in the root,

rhizosphere and bulk soil microbiomes under control and drought conditions. c Shannon index of microbial communities in both root and rhizosphere samples. Lowercase letters indicate the significant ($P < 0.05$ corrected using Bonferroni method, one-way ANOVA followed by LSD test) differences among genotypes. d Ternary plot of relative abundance-based families detected in the roots of Col-0, *gl2*, and *rsl2 rsl4*. Each circle represents each family. Different colors represent different families. e Accumulation of relative abundance of ASVs belonging to *Rhizobiaceae* increased with root hair density. Data are represented as mean (bar) ± standard error of mean (error bar). Different letters represent the significant ($p < 0.05$ corrected using Bonferroni method, one-way ANOVA followed by LSD test) differences among genotypes.

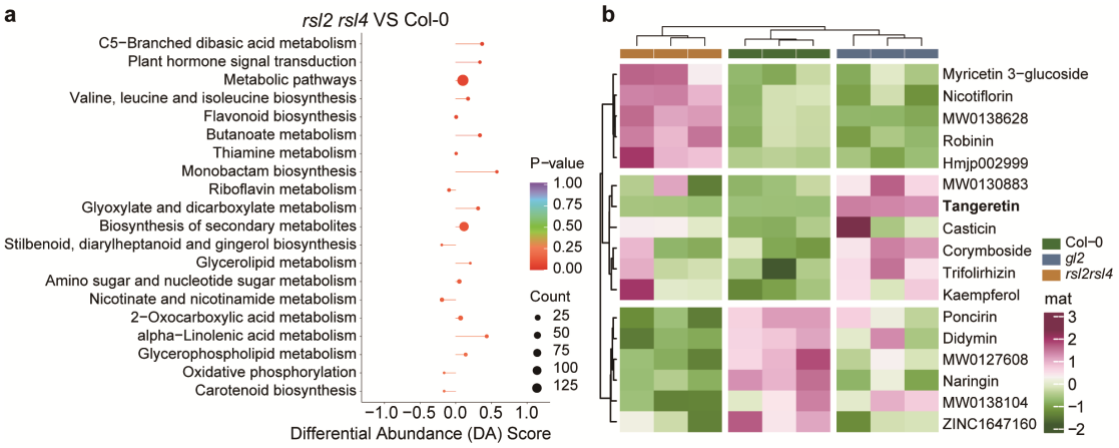


Supplementary Fig. 2. Manhattan plot showing the differentially abundant ASVs (DA-ASVs) enriched (Wilcoxon rank sum test, the dotted line indicates unadjusted $P < 0.05$) in the rhizosphere samples (**a-d**) or roots (**f-g**) of *rsl2 rsl4* and *gl2* or Col-0 under control and drought conditions (n=5 replicates for each individual group). Each dot and triangle represent each ASV. ASVs were colored according to the taxonomic families. Size of each dot or triangle represent the relative abundance of each ASV. Solid upward triangles indicate that ASV enriched in the roots of

mutant. Hollow downward triangles represent that ASV depleted in the mutants.



Supplementary Fig. 3 Microbial hub nodes (ASVs) within the root microbial community were identified in each co-occurrence networks under drought stress (related to Fig. 2d-f). Hub nodes were identified based on all three measurements of centrality (include degree, closeness centrality, and betweenness centrality). Different colors represent nodes belonging to different family. The hub ASVs were labeled. Yellow line: p = 0.1 based on a log-normal distribution fit.



Supplementary Fig. 4 Metabolome profiling of mutants affecting root hair densities. **a** Enriched pathways of DMs between the roots of *rsl2 rsl4* mutant and Col-0 under drought condition. The differential abundance (DA) score represents the changes in DMs within each pathway in the roots of *rsl2 rsl4* compared to Col-0. A score of -1 and 1 represent that all the DMs in the pathway were downregulated and upregulated, respectively. **b** Heat map showed the differences of relative content (log) of all differential flavonoids between the root hair mutant and Col-0.